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CHEMICAL STUDIES ON SAFFLOWER SEED AND ITS GERMINATION

STORAGE

BY

V. A. TAMHANE, M. Sc., M. Ag

Soil Physicist to Government, Bombay

(Formerly Senior Assistant to the Agricultural Chemist, Bombay)

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CHEMICAL STUDIES ON SAFFLOWER SEED AND ITS GERMINATION.

BY

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(Formerly Senior Assistant to the Agricultural Chemist, Bombay.)

[Received for publication on 30th May, 1922.]

I. Historical.

The germination of oil seeds has been often studied, but it cannot be said that the process can be, even yet, completely followed. The process by which the oil is converted into materials directly capable of being utilized by the seedling seems to be a very complicated one, largely dependent on the action of several enzymes. Most of the investigations made hitherto have been done on castor seed, a few on groundnut, but no worker has hitherto used the safflower seed (*Carthamus tinctorius*). This seed, however, has particular interest in that the oil in this seed is a very oxidisable one, and the phenomena connected with its germination may offer peculiar and interesting features. The seed, moreover, germinates very easily and very quickly, and is in every way suited for an investigation of this character.

There is no need to recall the earlier investigations on the germination of oil seeds. A full account of these can be found in the papers by Green and Deleano referred to below. The position in 1890, chiefly, be it said again, as a result of investigation on castor seed, was stated by Green[1] as follows :—

1. " The reserve materials in the endosperm of *Ricinus communis* consist chiefly of oil and proteid matters, the latter being a mixture of globulin and albumose.

2. " The changes during germination are partly due to enzyme action, there being three enzymes present in the germinating seed. One is a protease resembling trypsin, the second splits oil into fatty acid and glycerine, the third is a rennet enzyme.

[1] Green. *Proc. Roy. Soc.*, vol. 48 (1890), page 370.

3. " At least two of these and therefore presumably all of them are in a zymogen condition in the resting seed, and become active in consequence of the metabolic activity set up in the cells by the condition leading to germination, especially moisture and warmth.

4. " The changes caused by the enzymes are followed by others, due to the metabolism of the cells, these being processes of oxidation.

5. " The embryo exercises some influence on the latter, setting up as it developes a stimulus probably of a physiological description.

6. " The result of the various processes is to bring about the following decompositions :—

The proteids are, by the enzyme, converted into peptone and later into asparagin.

The oil is split by the glycerid enzyme into fatty acid and glycerine, the latter gives rise to sugar and the former to a vegetable acid which is soluble in water and in ether, is crystalline and has the power of dialysis.

7. " The absorption in all cases takes place by dialysis.

8. " The appearance of starch and of oil in the embryo or the young plant is due to a secondary formation and not to a translocation of either."

Further investigations by Green[1] added little to these results but, still working with castor seed, Deleano[2] has made a distinct advance, for he found and identified not only the enzymes detected by Green, but also a catalase, a peroxidase, and a reductase. It is probable that to one of these is due the oxidation noticed by Green (see para. 4 above) which he attributed to the direct effect of the cell metabolism.

The present paper attempts to determine how far some of these results are applicable to the safflower seed, the oil of which is of a very different type from that of castor and the matter, so far as it has been carried, will be discussed under the following headings :—

1. The composition of safflower seed in the resting condition.
2. The changes in the composition during germination.
3. The lipase of germinating safflower seed.
4. The oxidase of germinating safflower seed.
5. Summary and conclusions.

II. The composition of safflower seed in the resting condition.

The investigations, of which an account follows, were conducted with seed grown near Poona and at Bijapur in the Deccan, both considerable

[1] Green. *Proc. Roy. Soc.*, vol. 77 B (1905), page 85.
[2] Deleano. *Centr. Bakt.* (2), vol. 24 (1909), page 130.

centres for the growth of the crop. Good, well-formed and ripe seed only was selected for the experiments. The seed so selected derived from both the above centres, weighed on an average 0·05 gramme per seed, the individual seed varying little from this average. Its specific gravity is greater than water, and it just floated in 4 per cent. salt (NaCl) solution. As a rule, seed of this quality formed from half to two-thirds of good commercial samples.

After removal of the husk which forms approximately fifty per cent. of the seed, and which was not further investigated, the composition of the seed kernels is shown by the following figures calculated on the completely dried materials :—

	Poona seed per cent.	Bijapur seed per cent.
Ether extract	58·1	56·6
*Proteids	31·4	29·0
Woody fibre	0·7	1·5
Nitrogen-free extract	6·7	9·9
Ash	3·1	3·0
	100·0	100·0
*Containing nitrogen	5·0	4·6

The nitrogen-free extract was further investigated as regards the presence and quantity of starch, sugars, tannin and other constituents of possible importance.

Starch. A preliminary test with iodine indicated that, in ripe well-formed seed, starch is totally absent, and this was confirmed by careful microscopic examination. In thin sections, iodine gave no blue coloration with any element of the seed, while aleurone grains (stained brown with iodine) were very abundant.

Sugars. After extraction of the crushed seed with ether the residue, on treatment with water, gave a solution which did not reduce Fehling's solution. After inversion, however, with hydrochloric acid Fehling's solution was slowly reduced.

The source of the reducing power of the inverted solution was indicated by the following experiments: Five grammes of seed kernels were extracted with boiling water for ten minutes and cooled, the solution precipitated with basic lead acetate and the lead removed with sodium phosphate. The solution again gave no reaction with Fehling's solution. On inversion, however,

reduction was obtained equivalent to 0·37 per cent. of cane sugar on the original weight of seed. The sugar thus produced gave an osazone on treatment with phenyl hydrazine, crystallizing in a form exactly similar to that given by glucosazone when slowly cooled, but which melted at 185° to 187°C. On careful recrystallization from a mixture of pyridine and alcohol, large crystals were obtained, which melted after sudden heating at 190°C. A solution of this sugar rotating +1·5° before inversion showed a rotation of —1·0° after inversion with acid.

The nature of the sugar is thus not very clear, as the melting point of glucosazone should be over 205° C. The matter requires further investigation, but, in the meantime, it is clear that the safflower seed in the resting condition shows no reducing sugar but contains 0·37 per cent. (calculated as cane sugar) of a sugar which is non-reducing and is decomposed by acids with inversion of the rotation.

Glucosides. Careful tests were made for the presence of glucosides in the seed. Fifteen grammes of crushed safflower seed kernels were extracted with ether, and then steeped in absolute alcohol for twenty-four hours. The alcoholic extract was then evaporated, and the residue tested for glucosides with ammonium molybdate. No indication of glucosides was found, and the absence of any substances of this nature soluble in absolute alcohol is thus indicated.

A similar negative result was obtained by extracting crushed seed kernels with water, and precipitating the extract with basic lead acetate. On treating the precipitate with sulphuretted hydrogen a solution was obtained which again gave no tests for glucosides. Thus no glucoside soluble in water is present.

It appears, therefore, that glucosides soluble either in water or in absolute alcohol are not present in safflower seed in the resting condition.

Tannin. As the presence of small quantities of tannin would have seriously interfered with the later experiments on the germinating seed, it was essential to ascertain whether this substance was present in any noticeable proportion. Fifteen grammes of crushed kernels were therefore treated with ether, and the residue extracted with hot water. The solution was precipitated with lead acetate, the precipitate decomposed with sulphuretted hydrogen, and the resulting solution concentrated to a small volume. This solution now gave only a brown tinge with ferric chloride, no coloration with ferrous sulphate and no colour with ammoniacal solution of potassium ferricyanide. There is thus no tannin present in the resting stage of safflower seed.

In general, therefore, healthy, well-formed kernels of safflower seed consist of (1) fifty-five to sixty per cent. of ether extract, (2) about thirty per cent. of proteid, (3) about one per cent. of fibrous matter unattacked by dilute acid and alkali, and (4) from six to ten per cent. of nitrogen-free extract containing no starch, no glucosides, no tannin, no reducing sugar, and a small quantity of a non- reducing sugar resembling cane sugar, but whose character is not yet made out with certainty.

III. The changes in composition during germination.

Safflower seed germinates with great ease. The process was conducted in the first place in soil and the composition of the whole plant, including the seed kernel itself, determined at different stages.

The following stages were taken for examination in the first instance :—

(1) Resting seed.
(2) Seed testa just cracking.
(3) Radicle protruding.
(4) Lateral roots just appearing.
(5) Root system established.

When the stage desired was reached, the seeds were removed, washed to remove adhering soil particles, dried as far as possible with paper, and then completely dried in a vacuum desiccator.

The result of the analysis of the dried material at each stage is as follows:—

	Stage (1) Per cent.	Stage (2) Per cent.	Stage (3) Per cent.	Stage (4) Per cent.	Stage (5) Per cent.
Ether extract	58·1	57·5	56·0	10·1	5·3
*Proteids	31·4	31·6	33·3	31·6	34·1
Woody fibre	0·7	0·4	0·4	6·2	7·0
Nitrogen-free extract ..	6·7	7·7	7·5	41·9	40·1
†Ash	3·1	2·8	2·8	10·2	13·5
	100·0	100·0	100·0	100·0	100·0
*Containing nitrogen ..	5·0	5·1	5·3	5·1	5·4
†Containing Sand	0·3	0·04	0·04	0·6	0·6
Lime	trace	trace	trace	0 2	0·5
Phosphoric acid ..	1·5	1·5	1·5	1·5	1·6
Potash ..	1·4	1·4	1·4	4·1	5·7

The "ether extract" in stages (4) and (5) contained chlorophyll, and in obtaining the above figures this was removed by means of ninety-six per cent. alcohol from the original extract. The method is not however satisfactory and the figures for ether extract are doubtful in these cases, and in following tables the total crude extract only is recorded.

The above table shows that comparatively little change in general composition, as indicated by these analyses, takes place until the radicle has protruded, and the lateral roots are just forming. This is the point too, it appears, when mineral matter begins to be absorbed, for there is a considerable increase in the amount of lime and particularly in the amount of potash in the plant. To find the exact point at which the large change in composition (particularly in the reduction in the amount of oil and increase in the quantity of nitrogen-free extract) occurs, another stage was introduced in the next series of analyses between (3) and (4), while stage (2) (seed testa just cracking) was omitted.

Four pots were now sown with safflower seed in *pure sand*, distilled water being used for watering, and the seeds were removed and examined as before at the following stages :—

(1) Resting seed.
(2) Radicle protruding.
(3) Radicle one inch long.
(4) Lateral roots just spreading.
(5) Root system established.

The dried material at each stage gave the following figures on analysis :—

	(1) Per cent.	(2) Per cent.	(3) Per cent.	(4) Per cent.	(5) Per cent.
Ether extract	56·6	54·5	44·8	30·0	17·7
*Proteids	29·0	27·6	27·4	26·0	25·4
Woody fibre	1·5	1·7	2·0	3·1	5·3
Nitrogen-free extract ..	9·9	13·4	22·6	37·6	47·9
†Ash	3·0	2·8	3·2	3·3	3·7
	100·0	100·0	100·0	100·0	100·0
* Containing nitrogen.. ..	4·6	4·4	4·4	4·2	4·1
† Containing Sand	0·6	0·1	0·3	0·4	0·7
† Containing Lime	trace	trace	trace	trace	trace
† Containing Phosphoric acid ..	1·4	1·4	1·4	1·3	1·3
† Containing Potash ..	1·3	1·3	1·3	1·4	1·5

These figures indicate a much more regular and gradual reduction in the amount of ether extract than appeared in the previous ones and an almost corresponding increase in the amount of nitrogen-free extract and fibre taken together.

The changes in the amount and character of the sugars at these stages are interesting and are given in the following table :—

	(1) Per cent.	(2) Per cent.	(3) Per cent.	(4) Per cent.	(5) Per cent.
Total sugar	0·37	0·37	0·52	0·84	1·22
Reducing sugar (calculated as glucose)	nil	trace	0·33	0·29	0·21
Non-reducing sugar (calculated as cane sugar)	0·37	0·37	0·19	0·55	0·94

There is thus a general rise in the total amount of the sugars till germination is completed and the plant is capable of supplying its food from outside ; the reducing sugars on the other hand increase to an amount of 0·33 per cent. followed by steady reduction to 0·21 per cent., while the non-reducing sugar after a slight decrease in stage (3) rises rapidly to the end of the process.

Starch could not be detected at any stage of the germination.

The germination process is also characterized by a gradual increase in the solubility of the proteid matter, as is indicated in the following table :—

	(1) Per cent.	(2) Per cent.	(3) Per cent.	(4) Per cent.	(5) Per cent.
Total proteids	29·0	27·6	27·4	26·0	25·4
Insoluble proteids	27·9	26·3	23·7	15·2	7·9
Soluble proteids	1·1	1·3	3·7	10·8	17·4

There is thus a slight but regular and gradual fall in the total proteid matter. After stage (3) when the radicle is fully formed and before

lateral roots commence to develop, there is a sudden large increase in the soluble proteids which continues until germination is completed.

The characteristic features of germination of a highly oily seed like that of safflower are therefore (1) a gradual decrease in the oil content, (2) a gradual increase in the nitrogen-free extract (consisting largely of carbohydrates) which is very marked in the case of the sugars, (3) a gradual but slight loss of proteids, but a large increase in the proportion of soluble proteids. The changes indicated commence from the beginning, but become rapid after the radicle is well grown and just before lateral roots begin to be formed, and they all continue until the process is complete.

IV. The lipase of germinating safflower seed.

In the reactions taking place during the germination of all oily seeds hitherto examined, a lipase takes a considerable part. To ascertain whether this was also the case with safflower seed, one hundred grammes of seed was allowed to germinate on a sterilized wet cloth. After the radicle had grown an inch long (two to three days) the whole was crushed and extracted with 200 c.c. of 5 per cent. sodium chloride solution containing preservative, and filtered through cloth. This extract was then added to safflower oil kept at a constant temparature of 35°C. and the acidity produced determined from day to day, using a similar sample of extract which had been boiled as a control.[1]

In doing this great difficulty was experienced in preventing the growth of mould in the testa. The presence of 0·2 per cent. potassium cyanide solution, which was at first employed, seemed quite insufficient for this purpose, for in every case mould was visible to the naked eye after from seven to eleven days, and mycelia could be detected earlier on microscopic examination. Before, therefore, an experiment on the lines above indicated could give satisfactory results, an investigation had to be made as to the most suitable preservatives for preventing the growth of mould in connection with safflower seed and safflower seed extract.

Seed was therefore sown on sterilized blotting paper after being steeped in various strengths of copper sulphate solution, in 0·1 per cent. formalin solution, and in water saturated with chloroform. The results were as follows :—

(1) Seed steeped in $CuSO_4$ solution (0·5 per cent.) for 15 minutes. Mould appeared in three days.

[1] This method was essentially used by Green in studying the enzymes of castor seed. *Proc. Roy. Soc.*, vol. 48 (1890), p. 370.

(2) Seed steeped in $CuSO_4$ solution (1·0 per cent.) for 15 to 20 minutes. Mould appeared, but less vigorously.

(3) Seed steeped in $CuSO_4$ solution (2·0 per cent.) for 20 minutes. Mould appeared but only to a slight extent.

(4) Seed steeped in 0·1 per cent. solution of formalin for 10 to 15 minutes. Mould appeared in four days.

(5) Seed steeped in water saturated with chloroform for half an hour. Mould appeared in two to three days.

This is perhaps not unexpected, for the vitality of mould spores on seeds has been proved to be very great, even in presence of copper sulphate. The safflower seed[1], moreover, is well adapted to preserve them from injury by the steeping solution employed.

The secret, however, of the development of moulds was found to be germination on paper. If the use of paper was abandoned, and seeds previously treated with 2 per cent. copper sulphate solution were germinated in sterilized sand, no difficulty was ever found with the appearance of mould during germination, and no trace of its presence was ever detected by microscopic or other tests.

Apart entirely from the question of the development of moulds during germination, the method outlined above of extracting the enzyme was found to be unsatisfactory. It had been used by Green[2] in his investigations on the germination of castor seed, but it was abandoned in the present case because the potassium cyanide used for preventing the growth of moulds decomposed on standing in the dilute solution used, giving alkali which rendered doubtful any determinations of acidity.

Instead of this, the following method originally employed by Armstrong[3] was used. The seed, germinated to the extent desired, was dried and extracted with ether at the ordinary temperature. The residue was freed from ether by standing in the air, and the dried and powdered material which remained was tested for enzyme by allowing it to act on an emulsion of the oil with a little gum arabic solution. The extent of the acidity developed in the oil was used as a measure of the amount of lipase.

In the first series of experiments made to test this method with safflower seed in course of germination the following figures were obtained. Chloroform was added to the liquid every day, but no other sterilization was carried on. The acidity is represented by the number of cubic centimeters of

[1] Lesage. *Bull. Soc. Scientifique de l'ouest*, vol. 21, p. 129 (Rennes 1912).

[2] Green. *Proc. Roy. Soc.*, vol. 48 (1890), p. 370.

[3] Armstrong. *Proc. Roy. Soc*, vol. 78 (1906), p. 376.

centinormal potassium hydroxide required for neutralization, using phenolphthalein as indicator.

	(1)	(2)	(3)	(4)	(5)
	Control 10 c.c. oil only c.c.	10 c.c. oil, 0·5 grm. powder from seed c.c.	10 c.c. oil, 1·0 grm. powder from seed c.c.	10 c.c. oil, 1·5 grm. powder from seed c.c.	10 c.c. oil, 2·0 grm. powder from seed c.c.
Original acidity	2·0	7·5	13·0	15·7	19·8
Additional acidity—					
After first day	0·6	3·8	9·7	20·6	25·9
„ second day	0·4	9·2	14·3	28·6	31·7
„ third day	0·4	8·7	18·2	20·3	29·8
„ fourth day	0·3	8·5	21·5	16·2	25·7
„ fifth day	0·3	9·2	23·4	4·7	20·6
„ sixth day	0·2	3·0	26·1	3·6	6·9
„ seventh day ..	0·15	0·8	8·1	2·6	5·1
„ eighth day	nil	0·5	3·3	1·3	3·6
„ ninth day	nil	0·4	2·5	0·2	1·8
„ tenth day	nil	nil	1·2	nil	nil
„ eleventh day ..	nil	nil	nil	nil	nil
TOTAL ADDITIONAL ACIDITY ..	0·37	44·1	128·3	98·1	151·1

If we substract the original acidity in each case and also the acidity of the control on each day, we have for the first five days as follows :—

	(2) 0·5 grm. powder c.c.	(3) 1·0 grm. powder c.c.	(4) 1·5 grm. powder c.c.	(5) 2·0 grm. powder c.c.
Total acidity—				
After one day	3·2	9·1	20·0	25·3
„ two days	12·0	23·0	48·2	56·6
„ three „	20·3	40·8	68·1	86·0
„ four „	28·5	62·0	84·0	111·4
„ five „	37·4	85·1	88·4	131·7

From the second to the fourth day the amount of acidity produced is thus very closely proportional to the amount of seed powder used, and hence to the presumed amount of lipase present. After this time it becomes more

irregular, especially in No. 3, and it remains to account for this variation in the latter stages of the action. As it was suggested that this might be due to the fact that the chloroform did not entirely prevent the development of organisms, another similar test was made in which the whole apparatus was maintained completely sterile. Under these circumstances, the following figures were obtained. In the control flask, the powdered material was added, but the flask containing it was heated in boiling water for half an hour before the oil emulsion was added and the experiment commenced :—

	(1)	(2)	(3)	(4)	(5)
	Control 20 c.c. oil, 0·4 grm. powder previously heated c.c.	20 c.c. oil, 0·1 grm. powder c.c.	20 c.c.oil, 0·2 grm. powder c.c.	20 c.c. oil, 0·4 grm. powder c.c.	20 c.c. oil, 0·8 grm. powder c.c.
Original acidity	32·0	22·0	28·0	34·0	50·0
Additional acidity—					
After one day	11·0	11·0	21·0	24·0	57·0
„ two days	6·0	13·0	24·0	29·0	41·0
„ three „	2·0	7·0	10·0	15·0	29·0
„ four „	1·5	6·0	7·0	14·5	28·0
„ five „	1·0	4·0	5·0	9·0	15·0
„ six „	..	2·5	3·0	7·0	8·5
„ seven „	..	1·0	3·0	7·0	8·5
„ eight „	..	1·0	2·0	6·0	8·0
„ nine „	..	..	1·0	5·0	8·0
„ ten „	..	..	1·0	4·0	7·0
„ eleven „	..	..	..	4·0	4·0
„ twelve „	..	..	..	2·0	2·0
„ thirteen „	..	..	..	1·0	2·0
„ fourteen „	..	..	..	..	2·0
„ fifteen „	..	..	..	..	1·0
„ sixteen „	..	..	..	..	..
„ seventeen „	..	..	..	..	..
TOTAL ADDITIONAL ACIDITY ..	21·5	45·5	77·0	127·5	221·0

If, as in the last case, we substract the original acidity in each case, and also the acidity of the control on each day, we have as follows :—

	(2) c.c.	(3) c.c.	(4) c.c.	(5) c.c.
Total acidity—				
After one day	nil	10·0	13·0	46·0
„ two days	7·0	28·0	36·0	81·0
„ three „	12·0	36·0	49·0	108·0
„ four „	16·5	41·5	62·0	134·5
„ five „	19·5	45·5	70·0	148·5
„ six „	22·0	48·5	77·0	157·0
„ seven „	23·0	51·5	84·0	165·5
„ eight „	24·0	53·5	90·0	173·5
„ nine „	24·0	54·5	95·0	181·5
„ ten „	24·0	55·5	99·0	188·5
„ eleven „	24·0	55·5	103·0	192·5
„ fifteen „	24·0	55·5	106·0	199·5

There is thus a very close relationship between the acidity produced and the quantity of seed so far as the action can be due to an enzyme. There are, however, two points which appear from these experiments.

1. The whole of the acidification of the oil cannot be due to enzyme action. In both the experiments quoted there is some decomposition of the oil even where the ferment has been destroyed by heating or when no seed powder was present. In fact it would appear that the mere exposure of safflower oil to the air leads to a certain amount of decomposition, and that this fact may possibly be important in the germination of the seed. This matter demands further investigation.

2. The action of the enzyme is a terminable one, even in the presence of large excess of oil. In each case its increase of acidity arises to a maximum, generally on the second day, and then declines very gradually until action ceases after a time which varies according to the quantity of enzyme present. The larger the quantity of enzyme the longer the action lasts. It would appear, in fact, that the lipase enzyme is destroyed while acting.

So far we have dealt with the presence of a lipase fat-splitting enzyme in the germinating safflower seed, and have shown that such a lipase does exist

here as in other oily seeds in course of germination. It remained to see how far the quantity of this enzyme varied during the germination process. In fact it was desired to answer the question whether actual formation of a lipase enzyme took place during the early life stages of the safflower plant.

To do this, good sound seed was allowed to germinate in sterilized sand and the plants removed when the following stages were reached (*vide* page 228):—

1. Radicle just protruding.
2. Radicle one inch long.
3. Lateral roots just appearing.
4. Root system established.

In testing the relative amount of lipase a different method was employed from that previously described. The one now used is due to Tanaka[1] and consisted in treating the pressed seed with tenth-normal acetic acid, and drying the washed-out material at a temperature below 40°C. This gave a powder which could be dried and used for the determination of the lipase.

The actual working of the method was as follows : 0·4 grm of the dried powder thus obtained from each stage of germination was mixed with 10 c.c. of oil emulsified, along with 4 c.c. of water, the whole neutralized (to phenolphthalein) with tenth-normal caustic potash, and incubated at 35°C. In each case two such flasks were prepared, in one of which the enzyme had been destroyed by heating to the temperature of boiling water. By this means any acidification of the oil due to causes other than the enzyme can be eliminated, and the figures reported represent the difference between the acidity developed in the presence of active enzyme and of the same powder in which the enzyme had been destroyed. They are as follows :—

	Acidity produced in oil emulsion (Cubic centimeters of $\frac{N}{10}$ KOH for 0·4 grm. of dried powder) c.c.
Resting seed	0·1 (completed in 12 days).
Radicle just protruding	0·2 (completed in 11 days).
Radicle one inch long	5·0 (completed in 16 days).
Lateral roots just protruding	10·6 (completed in 22 days).
Root system established	8·3 (completed in 22 days).

[1] *Journal of the College of Engineering, Tokyo,* Imperial University (1910), vol. 5, no. 2, p. 25.

This result is exceedingly interesting when taken in conjunction with the changes in composition of the germinating seed already described (pages 228 and 229). The amount of active enzyme present in the resting seed is exceedingly small, and increases very little in the early stages of germination, or until the radicle has protruded from the seed. Then it rapidly increases as the radicle develops, reaching a maximum when the lateral roots (the true feeding rootlets) begin to develop, and then declines as the regular feeding roots are formed. This would indicate that in the earliest stages of germination in these oily seeds the plant is not dependent on the oil in the seed, but utilizes other materials present, and that only later is the oil a vital factor in the growth of the seedling. The nature of the material (zymogen) from which the lipase is formed has not yet been ascertained.

V. The oxidase of germinating safflower seeds.

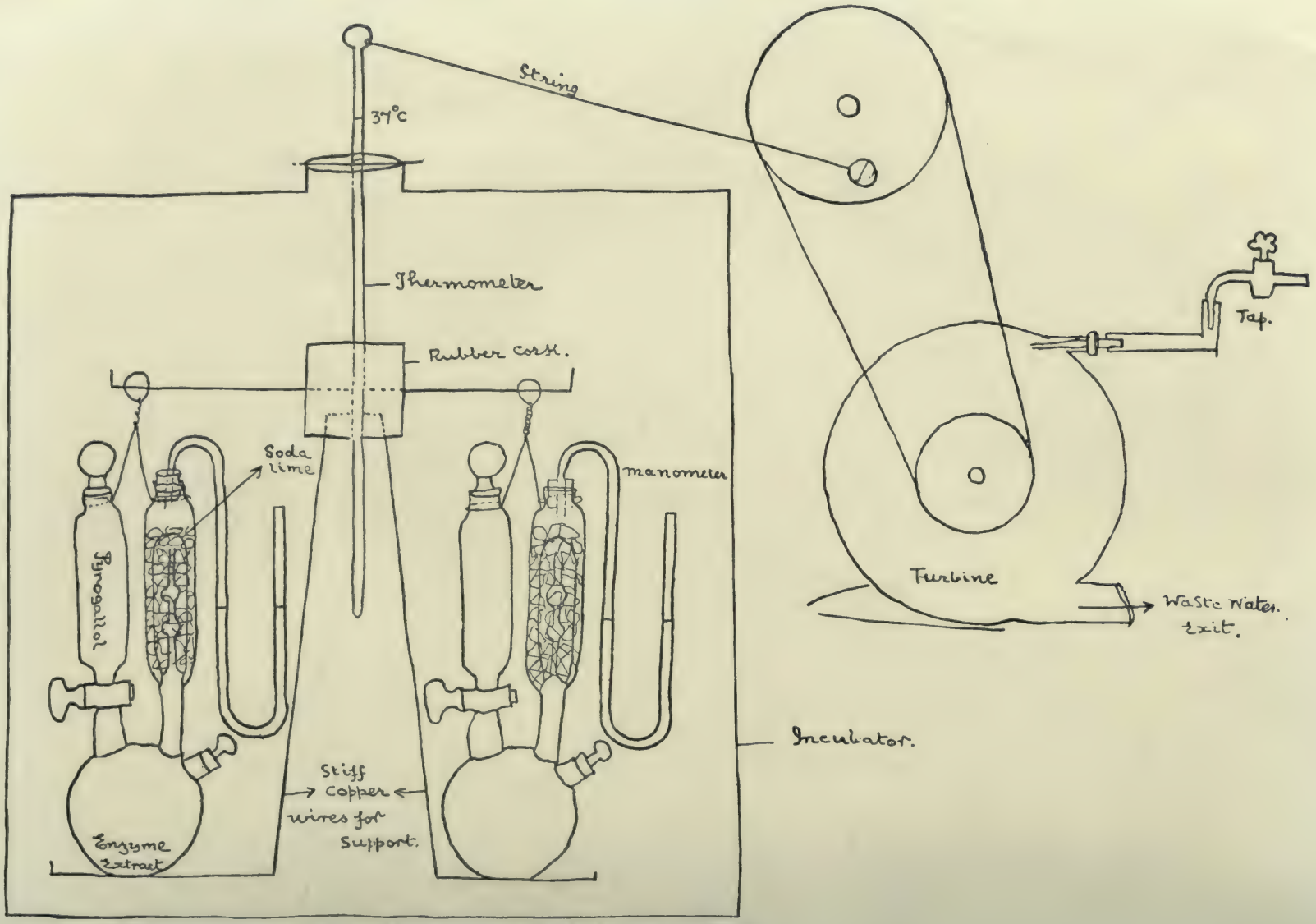
The study of the oxidases known to be present in germinating seeds and of the way in which these vary during the germination has not been undertaken, so far as I can ascertain, in the case of any oily seed such as that of the safflower[1]. Until comparatively recently a really satisfactory method of measuring the oxidase content of plant material was not known. But the perfecting of a method by Bunzel[2] which enables the relative amount of oxidase in a series of materials to be ascertained and which appears exceedingly satisfactory enables the development of oxidase to be followed with ease.

The presence of oxidase in the germinating seed was first shown by crushing the seed at any stage of the process, mixing with water, grinding up the wet mass, and filtering through linen. After washing and squeezing the mass remaining on the cloth, the filtrate was precipitated with three times its volume of alcohol, and, after filtration, the precipitate dissolved in water. The liquid thus obtained always gave a marked blue colour with an alcoholic solution of guaiacum resin which was not deepened by the addition of hydrogen peroxide. After heating the liquid to 90°C. this reaction could no longer be obtained. This reaction, characteristic of oxidases, removed any doubt as to their presence in germinating safflower seed.

The method of Bunzel for determining the amount of oxidase present in any plant material has, somewhat modified as to apparatus, been used throughout these experiments. It depends on the actual measurement of the

[1] Except by Deleano—see Bibliography and also Introduction.

[2] *U. S. Dept. Agri. Bureau of Plant Industry Bulletin* 238 (1912).



* The measurement of the oxidase content of plant juices by H. H. Bunzell—Bulletin No. 238, U. S. A. Department of Agriculture as modified by V. A. Tamhane.

Diagram Showing the oxidase apparatus.

amount of oxygen absorbed when the water extract of the ground material is mixed with a solution[1] of pyrogallol and kept at a constant temperature until absorption of oxygen ceases. The important things to secure are (1) a constant temperature, (2) an apparatus in which the carbon dioxide given off can be removed at once, and so the oxygen absorbed measured off directly, (3) a regular shaking of the apparatus during the whole process. These are secured by Bunzel, respectively, by an electrical thermoregulator, by an apparatus of his own devising which allowed the use of a definite quantity of pyrogallol solution, containing a receptacle for the removal of carbon dioxide by caustic potash as soon as formed, and carrying a manometer on which the absorption of oxygen could be directly measured, and by the use of an electrically driven shaking machine external to the thermostat.

Instead of these devices I employed a Hearson's incubator to maintain a constant temperature, a Schrœder CO_2 apparatus to which a manometer was attached as the reaction apparatus, and a small water turbine attached by an eccentric to a piece of glass rod to which the reaction apparatus was slung. The accompanying diagram shows the arrangement[2].

The whole process for determining the changes in the oxidase content in the germinating seed was as follows :—Good sound safflower seed was steeped in 1 per cent. copper sulphate solution for fifteen to twenty minutes and then sown in sterile sand kept moist with distilled water. When the seedling had grown to the stage desired, it was removed, washed free from adhering sand particles, and pressed lightly in blotting paper. About ten grammes of the seedlings were then weighed out and crushed along with a crystal of thymol as an antiseptic, ten cubic centimeters of water were incorporated with the pulp and the whole mass allowed to stand for half an hour. It was then pressed through cloth, and 4 c.c. of the extract was placed in the apparatus, with 8 c.c. of 1 per cent. pyrogallol solution and 4 c.c. of $2\frac{1}{2}$-normal caustic potash in their proper places. The manometer was then attached and the whole apparatus kept in the thermostat until it had reached a constant temperature of 37°C. with the stopper open to the air. When the temperature was constant, generally after about two hours, the pyrogallol solution was run into the extract, the stopper to the air closed, and the apparatus kept shaken with 25 to 30 complete oscillations per minute.

In about two hours the manometer began usually to show a fall in the pressure, a reading was made and fresh readings were taken at intervals as

[1] Palladin. *Ber. Deutsch. Bot. Gesells.*, vol. 24 (1906), p. 97.

[2] The diagram is from Vol 2, Part XIII, pp. 195-208 of the *Journal of the Indian Institute of Science, Bangalore*, where the apparatus was used after it was devised.

shown in the following tables, the whole test usually lasting about twenty-four hours. The results obtained were found to be constant in different tests with the same material, and closely proportionate to the amount of oxidase containing material employed. The constancy obtained is shown by the following figures :—

Experiment I. 2 c.c. oxidase extract taken in each of two cases (1) and (2). Experiment begun at 9 a.m. Constant temperature reached at 11 a.m. and pyrogallol added. Manometer readings (differences) in centimeters were obtained as follows :—

	(1) 2 c.c. extract	(2) 2 c.c. extract
	cm.	cm.
2 p. m.	0·2	0·2
4 „	0·5	0·6
6 „	1·0	1·2
11 „	1·0	1·2
8 a. m.	1·1	1·2

Experiment II. Details similar to the last, 4 c.c. extract however being used. Pyrogallol added at 11-30 a.m.

	(1) 4 c.c. extract	(2) 4 c.c. extract
	cm.	cm.
3 p. m.	0·25	0·32
6 „	0·60	0·65
9 „	1·30	1·52
12 Midnight ..	1·92	2·10
3-30 a. m.	2·12	2·50
6-30 „	2·23	2·60
30 „	2·24	2·60

Experiment III. 2 c.c. oxidase extract taken in (1), 4 c.c. oxidase extract taken in (2). Experiment begun at 9-30 a.m. Constant temperature reached at 11 a.m. and pyrogallol added. Manometer readings (differences) in centimeters were obtained as follows :—

	(1) 2 c.c. extract	(2) 4 c.c. extract
	cm.	cm.
2 p.m. ..	0·1	0·2
4 „ ..	0·2	0·5
6 „ ..	0·2	0·7
9 „ ..	0·3	0·7
10-30 p.m. ..	0·3	0·8
8 a. m.	0·4	0·8

Experiment IV. Details and times similar to the last.

	(1) 2 c.c. extract	(2) 4 c.c. extract
	cm.	cm.
1-30 p. m. ..	0·15	0·25
3-30 „ ..	0·20	0·50
5-30 „ ..	0·35	0·65
8-45 „ ..	0·40	0·70
11-30 „ ..	0·40	0·70
7-0 a. m. ..	0·40	0·70

Experiment V. Details similar to the last, except that in (2) four times the amount of oxidase extract was used than that employed in (1). Pyrogallol added at 1 p.m.

	(1) 2 c.c. extract	(2) 8 c.c. extract
	cm.	cm.
5-30 p. m. ..	0·10	0·44
8-30 „ ..	0·41	1·36
11-30 „ ..	0·53	1·80
2-30 a. m. ..	0·65	2·30
5-30 „ ..	0·70	2·71
8-30 „ ..	0·71	2·71

As the method appeared to give constant results, and as the readings obtained were proportionate to the amount of extract taken, the method was applied to the determination of the relative amounts of oxidase at different stages of germination. As the action seemed to be complete after eighteen to twenty-four hours the reading after this time was taken in each case.

I. In the first series the germination was carried out at the room temperature of 27 to 30°C.

Condition of seed and seedling	Manometer reading (differences) after 18 to 27 hours	
	a	b (duplicate)
	cm.	cm.
Resting seed	nil	nil
6 hours' germination	nil	nil
12 do. do. (radicle just protruding)	0·30	0·35
18 do. do.	0·75	0·83
24 do. do.	0·90	0·85
36 do. do.	1·05	0·99
48 do. do.	2·81	2·42
72 do. do.	2·60	2·45
96 do. do.	1·19	1·25

In this case the stage of germination reached may be considered to be as follows :—

(*a*) After 12 hours the radicle is just protruding.

(*b*) After 48 hours the radicle is well developed but without lateral roots.

(*c*) After 72 hours the lateral roots are just commencing to spread.

(*d*) After 96 hours the root system is established.

This being so, the increase in the proportion of oxidase closely corresponds with the increase previously shown to occur with the lipase. The quantity is negligible or very small until the growth of the radicle is considerable ; it reaches a maximum just before the lateral roots begin to form and thereafter rapidly diminishes as the capacity for absorbing outside food is developed.

II. A second partial experiment at the same temperature gave similar results.

Condition of seed and seedling	Manometer reading when constant (after 18 to 27 hours)	
	a	b (duplicate)
	cm.	cm.
Resting seed	nil	0·20
6 hours' germination	0·20	0·20
12 do. do.	0·60	0·70
24 do. do.	0·82	0·89

The figures are closely similar to those in the previous experiment except that the development of oxidase commences a little earlier.

III. A third series of experiments was now undertaken in which the germination was carried out at a higher and more constant temperature, namely, at 37°C. in a thermostat. In all other respects the method was identical with that just described.

Condition of seed and seedling	Manometer reading (differences when constant after 18 to 27 hours)	
	a	b (duplicate)
	cm.	cm.
a. 6 hours' germination	nil	0·15
12 do. do.	0·61	0·72
18 do. do.	0·83	0·94
24 do. do.	1·20	1·11
36 do. do.	2·30	2·53
48 do. do.	2·81	2·95
72 do. do.	2·83	2·42
b. 6 hours' germination	0·20	0·10
12 do. do.	0·60	0·57
24 do. do.	1·15	1·28
72 do. do.	2·56	2·39

When germination was carried out at 37°C. the root system was established after 72 hours, and if the experiment was continued for 96 hours the seedlings begin to show signs of decay. From general growth the seedlings were about six hours ahead of those at the room temperature of 27° to 30°C.

The general conclusions are however the same in the two cases. There is no oxidase in the resting seed, but it appears in minute quantity as soon as the radicle is formed. The proportion increases rapidly during the growth of the radicle, reaching its maximum when the lateral roots are commencing to form. After this it gradually declines, and it would appear as if it was not necessary in anything like such large proportion during the normal later life of the plant. After germination is over its amount probably varies very much with the amount of plant food supplied, and this introduces another factor demanding separate investigation.

VI. Summary and conclusions.

The conclusions which are reached as a result of the experiments described may be summarized as follows :—

(1) The safflower seed contains reserve materials chiefly in the form of oil and proteid matter. In the resting seed there are no starch, no glucosides, and no tannin, and a small proportion of non-reducing sugars (0·37 per cent.) capable of inversion. This does not seem to be cane sugar, but its identification has not yet been definitely made.

(2) During germination the composition, so far as the relative amount of oil, proteids and nitrogen-free extract is concerned, changes little until the radicle protrudes from the seed. Changes then rapidly occur. The oil disappears and a corresponding increase in the nitrogen-free extract takes place. This increase in the latter is not due to the formation of starch, which appears to be absent throughout. On the other hand sugars appear, non-reducing sugars gradually increasing as the germination becomes complete. The reducing sugars appear to rise to a definite proportion and then vary little. The proteid matter is not very largely solubilized until after the radicle has grown to a considerable extent. When the lateral roots are about to form and after this stage it is rapidly converted into a soluble form.

(3) The lipase occurs, as was found by Green in castor oil seed, very largely in the form of a zymogen, which is converted into the active condition during the germination itself. The amount of active enzyme in the resting seed is in fact exceedingly small and increases very little in the early stages of germination or until the radicle has protruded from the seed. Then it rapidly increases as the radicle develops, reaching a maximum when the lateral roots

begin to appear and then declines as the regular feeding roots are formed. It would appear as if the plant is not dependent on the oil in the earliest stages of germination, but first utilizes other materials present. Only later is the oil a vital factor in the growth of the seedling.

The experiments cited lead also to the conclusion that all the acidification of the oil during germination is not due to enzyme action. What the other cause may be has not been so far ascertained. Further, the action of the lipase seems to be a terminable one and hence the effect produced, as a certain quantity is limited. Whether the enzyme is destroyed during its action, or its activity is limited by the products which it forms, is not yet clear.

(4) The oxidase either occurs entirely in the form of a zymogen or it is a direct product of the cell metabolism in the early stages of germination. It is not found in the resting seed, but appears in minute quantity as soon as the radicle is formed. The proportion increases rapidly during the growth of the radicle, reaching its maximum when the lateral roots are commencing to form. After this it gradually declines and it would appear as if it was not necessary in anything like such a large proportion during the later life of the plant. At any rate its proportion declines in the absence of a supply of outside plant food. It is interesting to note that during germination the rise in the amount of oxidase follows a little the decomposition of the oil, and the rise in the amount of nitrogen-free extract in the germinated seedling.

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